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Identification and characterization of an anti-pseudomonal dichlorocarbazol derivative displaying anti-biofilm activity

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ABSTRACT

Pseudomonas aeruginosa strains resistant towards all currently available antibiotics are increasingly encountered, raising the need for new anti-pseudomonal drugs. We therefore conducted a medium-throughput screen of a small-molecule collection resulting in the identification of the *N*-alkylated 3,6-dihalogenocarbazol 1-(*sec*-butylamino)-3-(3,6-dichloro-9*H*-carbazol-9-yl)propan-2-ol (MIC = 18.5 μg mL^{-1}). This compound, compound **1**, is bacteriostatic towards a broad spectrum of Gram-positive and Gram-negative pathogens, including *P. aeruginosa*. Importantly, **1** also eradicates mature biofilms of *P. aeruginosa*. **1** displays no cytotoxicity against various human cell types, pointing to its potential for further development as a novel antibacterial drug.

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that poses a particular risk to immunosuppressed individuals and other highly vulnerable patients such as those in intensive care units.[1,2] This pathogen is also the dominant cause of life-threatening chronic lung infections in cystic fibrosis (CF) patients.[3] In addition to its considerable level of intrinsic resistance towards a wide variety of antibiotics, *P. aeruginosa* has a remarkable ability to acquire additional resistance mechanisms. This has led to development of resistance towards all available classes of antibiotics.[4] Alarmingly, the number of new antibiotics in the pharmaceutical pipeline active against *P. aeruginosa* is very limited.[2,5] Consequently, there is an urgent need to find new compounds with anti-pseudomonal activity.

To identify novel anti-pseudomonal compounds, we conducted a medium-throughput screening of a small compound library comprising 23,909 commercially available compounds.[6–8] The selection of compounds was based on (i) chemical diversity, (ii) druglike properties (Lipinski rule of five compliant), (iii) exclusion of unstable chemical groups and known toxicophores. All compounds were purchased from multiple commercial suppliers. Compounds that caused >90% growth inhibition were selected and retested (fresh sample), after which the most promising compound, 1-(*sec*-butylamino)-3-(3,6-dichloro-9*H*-carbazol-9-yl)propan-2-ol, designated as compound **1** (Fig. 1), was selected for further characterization.

Abbreviations: CF, cystic fibrosis; CLSM, confocal laser scanning microscopy; MBC, minimal bactericidal concentration; MBIC, minimal biofilm inhibitory concentration; MHB, Mueller-Hinton Broth; MIC, minimal inhibitory concentration; TSB, Tryptic Soy Broth.

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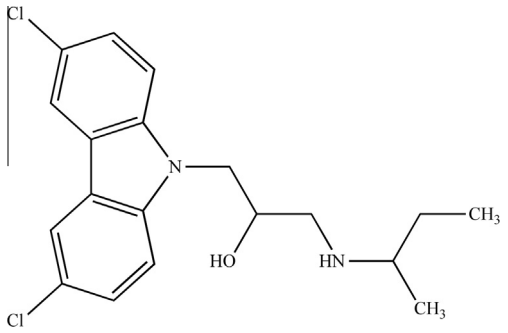


Figure 1. Schematic structure of compound **1**. Compound **1** was purchased from ChemBridge.

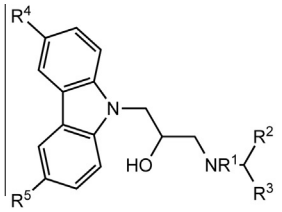
1 contains a carbazole motif, which is found in many pharmacologically important products. Besides antibacterial activity,[9–11] a variety of carbazole derivatives have been reported with antiviral,[12,13] antifungal,[14,15] anticancer,[16–18] anti-inflammatory,[19] antimalarial,[20,21] antidiarrhoeal,[22] mosquitocidal,[23] immunosuppressive[24] and neuroprotective[25] activities.

Commercially available chemical analogues of **1** were selected and purchased (compounds **2**–**8**, Fig. 2). The minimal inhibitory concentration (MIC) of these compounds was determined against *P. aeruginosa* grown in 1:20 Tryptic Soy Broth (TSB) (Table 2) as described previously.[26] The lowest compound concentration resulting in the absence of bacterial growth was considered as the MIC. None of the tested analogs showed better antibacterial activity against *P. aeruginosa* compared to the original hit compound **1** (MIC of 18.5 μg mL^{-1}, Fig. A.1). The minimal bactericidal concentration (MBC, concentration resulting in absence of bacterial regrowth after dilution in Mueller-Hinton Broth (MHB)) of **1** against *P. aeruginosa* is 18.5 μg mL^{-1}, indicating that this compound acts bactericidal. The MIC of **1** against a *P. aeruginosa* efflux mutant YM64 lacking all four major *mex* operons for multidrug efflux pumps[27] is 4.63 μg mL^{-1}, which is significantly lower compared to its corresponding wild-type strain YM (MIC of 18.5 μg mL^{-1}) (Table 1). This result indicates that **1** probably has to cross the *P. aeruginosa* outer membrane to exert its action.

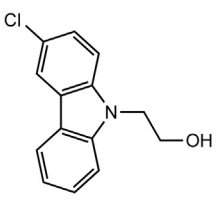
The susceptibility of several clinical isolates obtained from CF and non-CF patients towards **1** was tested. These strains were as susceptible towards **1** as the wild-type strain used in our study (Table 3). In addition, isolates resistant towards specific clinically used antibiotics did not show cross-resistance towards **1**. These results indicate that **1** is active against strains adapted to *in vivo* conditions, including antibiotic treatment regimes.

In vivo, *P. aeruginosa* is well known to form biofilms, multicellular structures of cells embedded in a self-produced extracellular matrix.[28,29] The bacterial cells residing in these biofilms are significantly less susceptible to antibiotics and host defenses. This complicates treatment and often leads to chronic and recurrent infections.[29,30] We assessed whether **1** is capable of preventing biofilm formation in vitro. The minimal biofilm inhibitory concentration (MBIC, concentration with 100% biofilm inhibition) was determined against biofilms grown on polystyrene pegs using the Calgary biofilm device (Nunc-Immuno TSP, VWR International).[31] The results show that **1** completely prevents biofilm formation of *P. aeruginosa* at 18.5 μg mL^{-1} (Table 4). To further evaluate the effect of **1** on biofilm formation on clinically relevant implant material, the formation of *P. aeruginosa* biofilms on smooth titanium discs (10 mm diameter, 2 mm thick) in the presence of **1** was investigated.[32] The results show that **1** is capable of preventing biofilm formation of *P. aeruginosa*, at a concentration as low as 3.7 μg mL^{-1}. The total area fraction, defined as the percentage of surface covered by bacteria, was reduced by approximately 50% upon addition of **1**. Biofilms formed on titanium discs in the presence of **1** comprised a greater proportion of dead cells within the biofilm compared to the control experiment. This was most evident at the highest concentration of **1** (12 μg mL^{-1}) (Figs. 3 and A.2). Taken together, these microscopy results clearly show that **1** prevents biofilm formation of *P. aeruginosa* on titanium discs. Next, we tested the capability of **1** to eradicate mature biofilms of *P. aeruginosa* grown on polystyrene pegs using the Calgary biofilm device.[31] Treatment of biofilms with 4.63 μg mL^{-1} or higher concentrations of **1** resulted in a drastic decrease of culturable cells within the biofilm, which demonstrates the capability of **1** to eradicate established biofilms (Fig. 4).

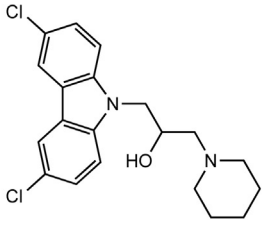
The effect of **1** was subsequently evaluated on the inhibition of growth[26] and biofilm formation[31] of other clinically relevant bacteria listed in Table 1. *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis* were selected as they are frequently involved in biofilm-associated infections.[29] Additionally, the efficacy of **1** was evaluated on the obligate anaerobic pathogen *Porphyromonas gingivalis*, which plays an important role in the initiation of periodontitis, a biofilm-associated infection of the gum eventually leading to tooth loss.[33] Results shown in Table 4 indicate that **1** is capable of inhibiting both planktonic and biofilm growth of other clinically important Gram-negative bacteria such as *E. coli* and *P. gingivalis* at concentrations well below those active against *P. aeruginosa*. **1** is as potent as the reference antibiotic chlorhexidine (MIC = 4 μg mL^{-1}) against *P. gingivalis*, but displays lower activity than ofloxacin (MIC = 0.1 μg mL^{-1}) on *E. coli*. Furthermore, **1** has a bacteriostatic and biofilm inhibitory effect on



(2) R^1: CH_3; R^2: H; R^3: CH_2OH; R^4: Cl; R^5: Cl
(3) R^1: CH_2CH_2OH; R^2: H; R^3: CH_2OH; R^4: Cl; R^5: Cl
(4) R^1: H; R^2: H; R^3: tetrahydrofuran; R^4: Cl; R^5: Cl
(5) R^1: CH_2CH_3; R^2: H; R^3: CH_3; R^4: Cl; R^5: Cl
(8) R^1: CH_3; R^2: H; R^3: H; R^4: Br; R^5: Br



(6)



(7)

Figure 2. Schematic structure of *N*-alkylated 3,6-dihalogenocarbazoles represented by numbers **2**–**8**. Compounds **2**, **3** and **4** were purchased from Interbioscreen, compounds **5**, **6**, **7** and **8** from ChemBridge.

Table 1
Bacterial strains

Strain	Description[a]	Source or reference
P. aeruginosa PA14	Wild type, Km^R	Lee et al.[38]
P. aeruginosa YM	Wild type	Morita et al.[27]
P. aeruginosa YM64	*mexAB-oprM::FRT, mexXY::FRT, mexCD-oprJ::FRT, mexEF-oprN::FRT*	Morita et al.[27]
E. coli TG1	Wild type, [F′ *traD36 proAB lacZ ΔM15] supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5(rK - mK -*	Carter et al.[39]
S. aureus SH1000	Functional *rsbU* derivative of 8325-4 *rsbU*$^+$ (wild type strain cured of prophages)	O'Neill[40]; Horsburgh et al.[41]
S. epidermidis RP62A	Wild type, ATCC 35984	ATCC
P. gingivalis	Wild type, ATCC 33277	ATCC

[a] Km^R: kanamycin resistant.

Table 2
Minimal inhibitory concentration (MIC) of *N*-alkylated 3,6-dihalogenocarbazoles against *P. aeruginosa*

Compound	MIC (μg mL^{-1})
1	18.5
2	18.5–37
3	>74
4	18.5–37
5	37–148
6	18.5–74
7	74–148
8	18.5–37

Table 3
Minimal inhibitory concentration (MIC) of **1** on *P. aeruginosa* clinical isolates obtained from cystic fibrosis (CF) and non-CF patients

Clinical isolate	Resistance profile	MIC 1 (μg mL^{-1})
Non-CF isolates		
PA08	Ticarcillin	18.5–37
PA53	Ticarcillin	18.5
PA129	Piperacillin	18.5–37
PA346	Ticarcillin, piperacillin	18.5–37
PA424	Ticarcillin	18.5–37
CF-isolates		
PA1250	Ticarcillin, cefepime	18.5
PA1255	Ticarcillin, piperacillin, ceftazidime, cefepime	18.5–37
PA1256	/	9.25–18.5
PA1270	/	18.5
PA1271	Ticarcillin	18.5–37

Table 4
Minimal inhibitory concentration (MIC) and minimal biofilm inhibitory concentration (MBIC) of **1** against *P. aeruginosa*, *E. coli*, *S. aureus*, *S. epidermidis* and *P. gingivalis* strains

Strain	MIC 1 (μg mL^{-1})	MBIC 1 (μg mL^{-1})	Growth medium
P. aeruginosa PA14	18.5	18.5	1:20 TSB
E. coli TG1	4.63	2.22[a]	1:20 TSB
S. aureus SH1000	9.25	9.25	MHB
S. epidermidis RP62A	9.25	9.25	MHB
P. gingivalis ATCC 33277	4.63	5.11	1:20 TSB

[a] $MBIC_{90}$.

both Gram-positive *S. aureus* and *S. epidermidis* strains. **1** shows comparable activity with the reference antibiotics ciprofloxacin (MIC = 6.25 μg mL^{-1}) and gentamicin (MIC = 12.5 μg mL^{-1}) against *S. aureus*. *S. epidermidis* is more susceptible to ciprofloxacin (MIC = 1.56 μg mL^{-1}) compared to treatment with **1**. The capability

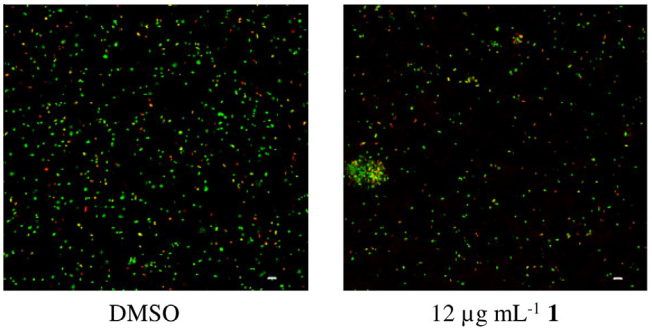


Figure 3. Representative CLSM micrographs of *P. aeruginosa* treated with **1**. The extent of the titanium surface that is covered by bacteria in a thin optical section at full width half maximum is shown after treatment with 0 and 12 μg mL^{-1} of **1**. Live cells were stained with CYTO®9 (green) and dead cells were stained with propidium iodide (red). Scale bar = 5 μm. Each sample was analyzed in triplicate.

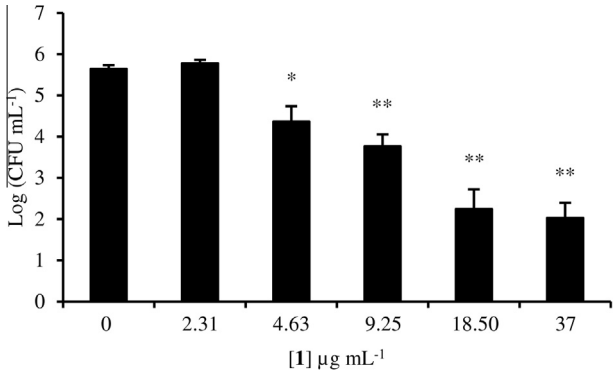


Figure 4. Compound **1** eradicates *P. aeruginosa* biofilms. 24 h old biofilms of *P. aeruginosa* were subjected to a 24 h treatment with different concentrations of **1**. Subsequently, biofilm cells were disrupted by sonication, diluted and plated out after which the number of surviving cells was determined. This experiment was repeated independently 3 times, error bars represent standard error of the mean (SEM). $^*P < 0.05$ and $^{**}P < 0.005$.

of **1** to target a wide range of bacterial species makes it a promising starting point for the development of a novel broad-spectrum antibiotic. Broad-spectrum agents are of particular importance in the treatment of acute life-threatening infections where immediate action is required and identification of the disease-causing pathogen is not feasible[34] or in case of polymicrobial infections.[35] A possible disadvantage of such a broad-spectrum agent is the higher chance of resistance development compared to narrow-spectrum compounds.[34] A previous, unrelated screening campaign identified **1** as a candidate antifungal.[15] Growth-inhibiting activity against bacterial as well as fungal pathogens could indicate that **1** targets an essential cellular process that is conserved among both prokaryotic and eukaryotic microbes.

As limited human cellular toxicity is an important feature for a compound with such a broad spectrum of action, the toxicity of **1** was evaluated on several human cell types. Cytotoxicity of **1** was tested on monolayer cultures of human osteoblasts, mesenchymal stem cells and endothelial cells, employing standard procedures as described previously.[36] While 2 h of exposure to 4.63 μg mL^{-1} of **1** results in >80% viability for osteoblasts and mesenchymal stem cells, these conditions are lethal to microvascular endothelial cells (data not shown). However, the tube formation potential of aortic endothelial cells is not negatively affected (Figs. 5 and A.3), indicating that more differentiated endothelial cells in the tube-assembled stage are less susceptible to the potential toxic effects of **1** compared to endothelial cells in the non-assembled stage in monolayer culture. The observed difference could also be due to the use of different types of endothelial cells in the two assays,

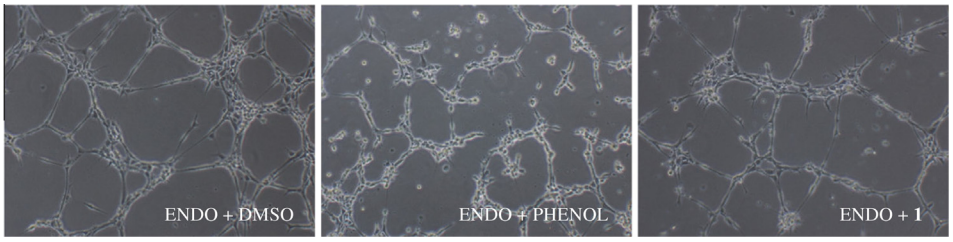


Figure 5. Tube formation potential of endothelial cells is not affected by **1**. Human endothelial cells were cultured in M200 medium with low serum growth supplement (ENDO) in the presence of 0.5% DMSO (control treatment), 0.05% phenol (positive cytotoxic control) or 4.63 μg mL^{-1} of **1** for 4 h. Average tube length is not inhibited by **1**, whereas 0.05% phenol causes a negative effect on tube formation (P <0.05).

as endothelial cells from different sources are functionally different and could therefore behave differently. Moreover, various carbazoles have previously been shown to possess low cellular toxicity at 80–100 μM,[37] pointing to the possibility to further develop **1** into a non-toxic novel antipseudomonal biofilm drug and a broad-spectrum antibacterial and antifungal agent.

In summary, we here demonstrate that a non-toxic dichlorocarbazol derivative displays anti-pseudomonal activity both on planktonic and biofilm cultures. Furthermore, **1** inhibits growth of several other clinically important pathogens making **1** an interesting starting point for the development of a broad-spectrum antimicrobial agent.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2014.10.039.

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31. *MBIC determination*. Bacterial cells from an overnight culture were diluted 1:200 (*P. aeruginosa*), 1:100 (*E. coli* and *Staphylococcus* species) or 1:10 (*P. gingivalis*) into fresh growth medium. *P. gingivalis* was cultured in an anaerobic jar ($H_2/CO_2/N_2$ 5%:5%:90%, Anoxomat AN2OP, Mart® Microbiology BV, Drachten, The Netherlands). Biofilms were allowed to form for 24 h (*E. coli, P. aeruginosa* and *Staphylococcus* species) or 72 h (*P. gingivalis*) on polystyrene pegs and were quantified as described previously.[42] For *E. coli* and *P. gingivalis*, MBIC90 (concentration resulting in 90% biofilm inhibition) values were determined. Prevention of *Staphylococcus* biofilm growth was quantified by determining the lowest concentration of compound that completely inhibits biofilms from seeding fresh growth medium as described previously[40,41] and biofilms were allowed to form for 24 h. *Determination of effect on established biofilms*. The effect on established *P. aeruginosa* biofilms was evaluated by subjecting 24 h old biofilms grown on polystyrene pegs to treatment with different concentrations of **1** during 24 h at 37 °C. Subsequently, biofilm cells were recovered by means of sonication after which the number of surviving cells was determined by plate counting as described previously.[43]
32. *Microscopic evaluation of biofilm formation on smooth titanium dics*. Overnight *P. aeruginosa* cultures were diluted into fresh TSB to 1×10^5 cells mL^{-1} and transferred to a 6-well plate containing a titanium disc and 0, 3.7 and 12 μg mL^{-1} of **1**, respectively. Subsequently, biofilms were allowed to form for 19 h at 37 °C with agitation. Biofilm formation was analyzed using confocal laser scanning microscopy (CLSM, Leica TCS SP5). In addition, viability of the

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